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Perspective

In Vitro and in Vivo Induction of Cytochrome P450: A Survey of the Current Practices and Recommendations: A Pharmaceutical Research and Manufacturers of America Perspective[S]

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ABSTRACT:

Cytochrome P450 (P450) induction is one of the factors that can affect the pharmacokinetics of a drug molecule upon multiple dosing, and it can result in pharmacokinetic drug-drug interactions with coadministered drugs causing potential therapeutic failures. In recent years, various in vitro assays have been developed and used routinely to assess the potential for drug-drug interactions due to P450 induction. There is a desire from the pharmaceutical industry and regulatory agencies to harmonize assay methodologies, data interpretation, and the design of clinical drug-drug interaction studies. In this article, a team of 10 scientists from nine Pharmaceutical Research and Manufacturers of America (PhRMA) member companies conducted an anonymous survey among PhRMA companies to query current practices with regards to the conduct of in vitro induction assays, data interpretation, and clinical induction study practices. The results of the survey are presented in this article, along with reviews of current methodologies of in vitro assays and in vivo studies, including modeling efforts in this area. A consensus recommendation regarding common practices for the conduct of P450 induction studies is included.

The convergence of recent advances in molecular biology and genomics, higher throughput chemical synthesis, and automated high-throughput in vitro enzyme and cell-based assays to assess biological activity has led to shorter lead identification and optimization times in drug research. However, these breakthroughs have not yet translated into success in drug development. Surveys suggest that in the last several years, the number of New Drug Applications submitted to regulatory agencies has declined and that the poor success rate during drug development is to some extent due to late-stage failures. It is important that novel and innovative approaches are used for assessing the risks and benefits of new molecular entities (NMEs) early in the research and development life cycle to minimize late-stage attrition.

Based on a survey conducted in 2004, the major factors for compound attrition during clinical development are lack of efficacy, toxicity, and safety, and suboptimal pharmacokinetics and/or bioavailability, with the remaining failures due to financial and/or commercial reasons (Kola and Landis, 2004). In that survey, one notable observation was the reduction of compound attrition due to pharmacokinetic and/or bioavailability issues from pre-1991 to the period be-

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ABBREVIATIONS: NME, new molecular entity; P450, cytochrome P450; DDI, drug-drug interaction; RIF, rifampicin; PhRMA, Pharmaceutical Research and Manufacturers of America; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; UGT, uridine diphosphate glucuronosyl transferase; FXR, farnesyl X receptor; PPAR, peroxisome proliferator-activated receptor; VDR, vitamin D receptor; Nrf2, nuclear factor erythroid 2-related factor 2; LBD, ligand binding domain; hPXR, human PXR; SR12813, tetra-ethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; SAR, structure-activity relationship; EC_{50}, concentration to reach half the maximal induction effect; bp, base pair; %Act, percentage of activation; [Ind], inducer concentration; E_{max}, maximal in vitro induction effect; E_{min}, background level of catalytic activity; AUC, area under the curve; TCDD, 2,3,7,8-tetrachlorobenzo-p-dioxin; 3-MC, 3-methylcholanthrene; BNF, β-naphthoflavone; CITCO, (6-(4-chlorophenyl)imidazo[2,1-β][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime); ITS, insulin, transferrin, and selenium as supplements in hepatocyte culture medium; DMSO, dimethyl sulfoxide; MDR-1, multidrug resistance-1 gene encoding P-glycoprotein expression; IVIVC, in vitro-in vivo correlation; MTT, 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; fu, fraction unbound in blood (plasma); CL_{int}, intrinsic clearance; RIS, relative induction score; F_2, concentration of inducer leading to a 2-fold increase of CYP3A4 mRNA in hepatocytes in vitro; NOEL, no observed effect level.

TABLE 1

A representative set of clinical drug-drug interactions due to P450 induction

P450	Inducer (Substrate)	Clinical Effect	Reference
CYP1A2	Omeprazole (caffeine)	39% Increase in exhalation of $[^{14}C]CO_2$ in CYP2C19 poor and intermediate metabolizers compared with 12% increase in extensive metabolizers	Rost et al., 1992
CYP2B6	Rifampicin (bupropion)	Apparent clearance increased from 2.6 l/h/kg to 7.9 l/h/kg	Loboz et al., 2006
CYP2C9	Rifampicin (celecoxib)	AUC decreased by 64%	Jayasagar et al., 2003
CYP2E1	Alcohol (chlorzoxazone)	Total plasma clearance 73% higher in alcoholics	Girre et al., 1994
CYP3A4	Efavirenz (simvastatin)	Simvastatin-acid AUC decreased 58%, and HMG-CoA activity decreased 60%	Gerber et al., 2005
CYP3A4 and -2C8	Rifampicin (repaglinide)	AUC decreased by 57%; blood glucose decremental AUC (0–3 h) reduced from 0.94 to −0.23 mmol/l	Niemi et al., 2000
Multiple	Lopinavir/ritonavir		
CYP1A2	(caffeine)	Urinary metabolite ratios increased by 43%	Yeh et al., 2006
CYP2C9	(warfarin)	AUC decreased by 1.4-fold	
CYP2C19	(omeprazole)	Omeprazole/5-hydroxyomeprazole ratio decreased 2.7-fold	
CYP3A4	(midazolam)	Apparent clearance decreased by 12-fold	

tween 1991 and 2001. This result could be, in part, due to the development and adaptation of in vitro drug metabolism assays using human biomaterials in the lead optimization stage, thereby minimizing the potential for unacceptable disposition profiles in humans. However, there is still a need to develop better predictive models for further minimization of compound attrition due to pharmacokinetic issues.

Metabolism by the cytochrome P450 (P450) monooxygenases is an important determinant of the pharmacokinetic disposition of a majority of marketed drugs. The expression of this diverse group of enzymes varies widely in the human population. These enzymes are subject to inhibition, and some of them are subject to induction by xenobiotics, leading to pharmacokinetic drug-drug interactions (DDIs) between two or more coadministered drugs (Tucker et al., 2001). The earliest observations that the drug-metabolizing enzymes are inducible were made in animals. Polyaromatic hydrocarbons and phenobarbital were found to increase the catalytic activities of liver microsomal enzymes (Richardson et al., 1952; Conney et al., 1956). In humans, many drugs, such as rifampicin (RIF), phenobarbital, and carbamazepine, are known to cause clinically significant decreases in the systemic exposure of certain coadministered drugs, which have been shown to be due to induction of the P450 isoforms responsible for the metabolism of those drugs. A representative set of examples is summarized in Table 1. In addition to drugs, ethanol, cigarette smoking, and herbal products such as St. John's Wort are also capable of inducing certain P450 enzymes. Several review articles have been published that detail the biochemical mechanisms involved in P450 induction and the clinical importance of P450 induction (Bresnick et al., 1984; Park et al., 1996; Ma and Lu, 2007).

P450 induction can potentially lead to decreased systemic exposure of the inducing compound, if it is metabolized by the induced enzyme (autoinduction, e.g., carbamazepine), or decreased systemic exposure of a coadministered compound that is subject to metabolism by the induced enzyme. The magnitude of in vivo-inductive effects varies widely among the different P450 isoform-mediated activities, i.e., from minor effects seen with CYP1A2 inducers to major effects seen with CYP3A4 inducers (Table 1). Enzyme induction can potentially lead to subtherapeutic concentrations of the affected drug. In addition, if the affected drug is metabolized to an active or toxic metabolite, the pharmacological and toxicological outcomes in the induced state will be different from that in the uninduced state. Hence, it is important to assess the potential for P450 induction early in the research and development cycle and to plan appropriate clinical drug-drug interaction studies.

Recognizing the importance of drug-drug interactions in the overall clinical safety profile of medications, the U.S. Food and Drug Administration issued a *Guidance for Industry* (1997) for in vitro studies, a *Guidance for Industry* (1999) for in vivo studies, and a Draft Guidance for Industry (2006). The Pharmaceutical Research and Manufacturers of America (PhRMA) Drug Metabolism and Clinical Pharmacology Technical Groups published a White Paper on the conduct of in vitro and in vivo drug-drug interaction studies in 2003 (Bjornsson et al., 2003). In addition, Hewitt et al. (2007a,b) recently published two articles summarizing an informal survey and recommendations for the conduct of P450 induction assays. Pursuant to the Critical Path Initiative by the U.S. Food and Drug Administration (http://www.fda.gov/oc/initiatives/criticalpath/), PhRMA formed a Pharmaceutical Innovation Steering Committee, which is charged with working on various scientific topics of interest to the pharmaceutical industry in regard to Predictive Models of Safety, Efficacy, and Compound Properties. The present topic focuses on Predictive Models of Compound Properties, outlining work to assess the predictability of various in vitro experimental models currently used across the industry to predict drug-drug interactions due to P450 induction. The key objectives of this effort were as follows: 1) identify the current practices used by PhRMA member companies; 2) collate information on methods and models; 3) assess the success/failure of predictability based on current methods and models; 4) identify areas with the greatest need for better predictive methods and models; 5) stimulate interest and promote research for the development of better predictive methods; and 6) foster the development of general methodologies and frameworks that may decrease compound attrition during drug development.

The PhRMA Drug Metabolism Technical Group has formed an expert team from member companies to steer this effort. An anonymous survey, comprising 61 questions, was conducted to gather information about the current practices for nuclear receptor assays, immortalized hepatocyte assays, human hepatocyte assays, clinical induction studies, and new technologies. The responses for the survey were received from 14 PhRMA member companies, which included 10 large companies (>10,000 employees) and four medium-sized companies (1000–10,000 employees). Information gathered from this survey is presented in this article, along with a set of consensus recommendations surrounding the conduct of these assays and data

interpretation. Detailed survey data are presented as supplemental information associated with this article.

Nuclear Receptor Assays. The primary mechanism by which drugs or xenobiotics can cause enzyme induction is by the activation of gene transcription. Most commonly, a compound is considered an inducer if it activates a nuclear receptor, thereby causing increased expression of the receptor's target genes. The nuclear receptors that have the broadest ligand selectivity and are most commonly involved in the activation of transcription of drug-metabolizing enzymes and transporters are the aryl hydrocarbon receptor (A*h*R), pregnane X receptor [(PXR) NR1I2], and constitutive androstane receptor [(CAR) NR1I3]. Whereas the A*h*R is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim family, CAR and PXR are members of the nuclear hormone receptor superfamily. Target genes for these receptors of interest to the study of drug metabolism include various Phase I (e.g., members of the CYP1A, -2B, -2C, and -3A subfamilies) and Phase II enzymes [e.g., uridine diphosphate glucuronosyl transferases (UGTs), glutathione *S*-transferases, and sulfotransferases] and drug transporters including P-glycoprotein (Kast et al., 2002; Maglich et al., 2002; Madan et al., 2003; Donato et al., 2005; Girault et al., 2005; Hartley et al., 2006; Jigorel et al., 2006). Activation of other nuclear receptors such as the farnesyl X receptor (FXR), liver X receptor, peroxisome proliferator-activated receptors (PPARs), and the vitamin D receptor (VDR) has been associated with induction of genes relevant to drug metabolism. In addition, induction of Phase II enzymes and NAD(P)H:quinone oxidoreductase in response to antioxidants and chemopreventive chemicals can be mediated by a transcription factor known as nuclear factor erythroid 2-related factor 2 (Nrf2) (see Ripp, 2008, and references therein). However, because FXR, liver X receptor, PPAR, VDR, and Nrf2 have much narrower substrate specificity and activate a more limited set of genes, they are not typically associated with DDIs and therefore are not covered further in this article.

More recently, the term "xenobiotic-activated receptor" has been proposed to describe the receptors that are involved in the activation of transcription of genes responsible for the metabolism and disposition of drugs and xenobiotics, including P450s (Ma, 2008).

In Silico Models of Nuclear Receptors: Human PXR. PXR consists of an N-terminal DNA-binding domain and a C-terminal ligand binding domain (LBD). It forms a heterodimer with the retinoic X receptor (NR1B2). Several crystal structures of the human PXR (hPXR) with xenobiotic ligands have demonstrated that the hPXR binding site is unusually large and flexible. The structure of the hPXR ligand binding domain consists of a three-layered α-helical sandwich with a unique five-stranded antiparallel β-sheet and a very large cavity (Poso and Honkakoski, 2006). The large and flexible binding pocket explains why human PXR responds to a wide range of drugs, xenobiotics, and endogenous compounds, both varying in structure and molecular mass [e.g., paclitaxel (854 Da) and phenobarbital (232 Da)]. It is important to note that the nature of the ligand binding pocket allows compounds to bind in different configurations (three were observed for SR12813) (Watkins et al., 2001), although it appears that flexibility was reduced in the presence of the transcriptional coactivator SRC-1 (Watkins et al., 2003).

Detailed knowledge of the structure of PXR and the understanding of how ligands bind to PXR have resulted in attempts to develop a structure-activity relationship (SAR) for PXR binding or activation. Responses to the survey indicated that only two of the participating companies (14%) have evaluated quantitative structure-activity relationship and/or structure-based approaches to predict and/or attenuate PXR activity. The challenge thus far has been that it is difficult to use in silico models prospectively or to establish SAR, especially in attempts to apply models to new chemical space not included in the training sets. In addition, a lack of consistency has been observed between structure and activity. Several studies have been published applying modeling approaches to identify PXR agonists. Using literature data for EC_{50} values for 12 PXR ligands, a pharmacophore model for hPXR was developed (Ekins and Erickson, 2002). This model was also used to predict the binding affinity for molecules not in the model but known to be PXR ligands with different potencies. Based on the limited number of compounds assessed, we concluded that the model could be used as a filter to identify compounds with high hPXR activation potential. An example where a docking approach was used to study the SAR for PXR to a series of closely related analogs was published by Gao et al. (2007). In another recent article, Ung et al. (2007) used a combination of machine learning methods and support vector machines as a potential tool to identify novel PXR activators from structurally diverse compounds.

The same companies that evaluated PXR modeling approaches also applied in silico docking and energy minimization techniques for other nuclear receptors such as the A*h*R, estrogen receptor, VDR, PPAR, and glucocorticoid receptor. It was indicated that these approaches were useful and that guidance could be provided to medicinal chemists by identifying novel agonists and antagonists.

In summary, application of modeling approaches to identify agonists/antagonists of nuclear receptors is an emerging field in which more research is needed to evaluate the applicability of various strategies.

PXR Binding Assays. Several in vitro PXR binding assays have been described previously (Jones et al., 2002; Zhu et al., 2004), which are based on the displacement of a radiolabeled or fluorescently labeled high-affinity PXR ligand by test compounds. Assays are conducted with the purified PXR LBD. Interestingly, it has been reported that stability and solubility of the LBD are enhanced by coexpression with a fragment of the transcriptional coactivator SRC-1 (Jones et al., 2002).

Although in vitro binding assays can be used in high-throughput mode and can be automated relatively easily, the majority of companies (93%) do not routinely use these assays to assess the potential of compounds to cause enzyme induction. One company indicated that if binding assays are used, it is for mechanistic studies only. The lack of routine application of binding assays is most likely due to the lack of a strong correlation between receptor binding and transactivation. An extensive comparison between PXR binding and transactivation was conducted by Zhu et al. (2004). From 616 compounds tested, 8% demonstrated both high binding and transactivation, 9% demonstrated high binding but low transactivation, 82% were low in both assays, and 1% showed significant transactivation but low binding. The lack of correlation between binding and transactivation for some compounds was most likely explained by the fact that there is not always a linear correlation between the binding affinity and the downstream response generated by the binding because this correlation depends on the conformational change caused by the inducer (Zhu et al., 2004).

Nuclear Receptor Transactivation Assays. Of the survey respondents, 64% routinely use nuclear receptor transactivation assays to assess the potential of test compounds to cause enzyme induction. In the majority of cases PXR is being evaluated, whereas some companies also determine the activation of the A*h*R. In general, such determinations are performed in the drug discovery phase. Activation of CAR is not assessed by any company due to the current lack of availability of an in vitro assay.

Whereas cell-based transactivation assays are available to most companies to identify PXR agonists, the way data are expressed is variable. EC_{50} values are determined by the majority of companies,

but, in addition, data are expressed as a percentage of a positive control, and maximum stimulation is expressed as a percentage of a positive control or -fold increase above a positive control. Currently, 57% of the survey participants do not assess or interpret whether compounds are partial agonists in transactivation assays. One company assesses the E_{max} and ignores the EC_{50} value, whereas others do not consider partial versus full agonists.

Currently, the study of antagonism of hPXR by drugs and dietary components is an area of intense academic research. For example, sulforaphane, a biologically active phytochemical found in broccoli, represses hPXR activity in vitro at concentrations close to those achieved in humans (Zhou et al., 2007). In addition, the phytoestrogen coumestrol (Wang et al., 2008) and members of the azole family (e.g., ketoconazole) have been identified as PXR antagonists (Ekins et al., 2007). Although nuclear receptor antagonists have been observed by 50% of survey respondents, it is currently not clear how these data should be interpreted from a pharmaceutical industry perspective. A deeper understanding is needed to determine whether antagonism results in down-regulation of the basal expression level of PXR target genes and whether this effect could result in clinically relevant outcomes like toxicity or drug-drug interactions.

It is well established that species specificity exists with respect to gene response(s) to pharmaceutical agents. For example, rifampin is a potent inducer of CYP3A in rabbit and human liver, but it is a poor inducer of Cyp3a in rats (Moore et al., 2003). Conversely, the anti-glucocorticoid pregnenolone 16α-carbonitrile and glucocorticoid dexamethasone are potent inducers of Cyp3a in rats and mice but not of CYP3A in human liver. The species-specific induction of CYP3A/Cyp3a has been attributed in part to the differences in amino acid sequence in the ligand binding domain of PXR in different species. In contrast, across various species such as mice, rats, rabbits, and humans, the DNA binding domain of PXR is well conserved with >96% amino acid sequence homology. Due to species specificity in PXR response, it is not possible to use human PXR data to predict the likelihood that autoinduction will be found in safety studies performed in preclinical species. Therefore, some companies (17%) use, or are in the process of developing, PXR assays for preclinical species. Rat Pxr is of interest to many because rats are widely used in safety assessment studies, and repeat dosing of Pxr agonists could result in time-dependent decreases in exposure due to autoinduction.

PXR Assay Formats and Recommended Assay Conditions. PXR assays are generally conducted with carcinoma cell lines such as HepG2 or HuH7 transiently or stably transfected with PXR expression and reporter constructs (El-Sankary et al., 2001; Jones et al., 2002; Yueh et al., 2005; Sinz et al., 2006; Gao et al., 2007). As response elements in the reporter constructs, the CYP3A4 proximal promoter region [e.g., base pair (bp) −362 to +64 or bp −568 to +50] linked to the distal xenobiotic-responsive enhancer module (e.g., bp −7839 to −7208 or bp −7836 to 7106) is generally used. These regulatory elements are positioned just upstream of reporter genes such as luciferase, chloramphenicol acetyltransferase, or secreted placental alkaline phosphatase.

After challenging the transfected cell lines with test compounds for 24 to 48 h, PXR activation is determined by measuring reporter gene activity. The number of replicates and test compound concentrations used vary between the various companies and depend on the stage of drug development that the assay is conducted and the variability of the assay. Replicates vary from 2 to 6, and the number of test compound concentrations varied from 5 to 10. Most companies are using a 96-well-based plate format, although 24- or 384-well formats are also being used.

PXR Transactivation: Data Interpretation. PXR assay results have been used to generally rank order compounds (El-Sankary et al., 2001) or qualitatively categorize the potency of PXR activators (e.g., low, moderate, and high DDI potential) (Persson et al., 2006; Sinz et al., 2006). PXR activation data are often expressed as percentage of activation (%Act) compared with a positive control, where the "total signal" is the signal from the positive control (e.g., 10 μM rifampicin) and the "blank signal" is the signal from the solvent-treated wells (Sinz et al., 2006). Data are then plotted as percentage of activation versus log[inducer concentration] as described by eq. 1.

$$\%\text{Act} = \frac{\text{Compound Signal} - \text{Blank Signal}}{\text{Total Signal} - \text{Blank Signal}} \times 100\% \quad (1)$$

It is recommended to also calculate the activation data as a percentage of the positive control response to compare interday study results. Because cytotoxicity and solubility issues will be encountered with some compounds, EC_{50} curves cannot always be derived. In such cases, the maximum percentage of transactivation and the compound concentration at which this activation is measured are helpful in interpreting the induction potential of a compound (Sinz et al., 2006). It is recommended that knowledge about limits of cytotoxicity, metabolic stability, and solubility are considered when interpreting transactivation assay results.

Values of E_{max} and EC_{50} from in vitro data are typically calculated by nonlinear regression analysis of the -fold activation response above the vehicle control versus inducer concentration. A common equation used for transactivation assays is described by the sigmoidal E_{max} model (eq. 2) (El-Sankary et al., 2001; Ripp et al., 2006; Hariparsad et al., 2008).

$$\text{Effect} = \frac{E_{max} \times [\text{Ind}]^{\gamma}}{\text{ED}_{50}{}^{\gamma} + [\text{Ind}]^{\gamma}} \quad (2)$$

where E_{max} is the maximal in vitro induction effect, EC_{50} is the concentration to reach half the maximal in vitro induction effect, also referred to as the in vitro induction potency, [Ind] is the concentration of inducer in the in vitro assay, and γ is the Hill or sigmoidicity coefficient that accommodates the shape of the curve. Alternately, the sigmoidal dose-response Hill model has been used to describe induction data (eq. 3) (Persson et al., 2006; Kanebratt and Andersson, 2008).

$$\text{Effect} = E_{min} + \frac{E_{max} - E_{min}}{1 + \left(\frac{EC_{50}}{[\text{Ind}]}\right)^{k}} \quad (3)$$

where E_{max}, EC_{50}, and [Ind] are as described above, E_{min} describes the background level of the effect, and k is the slope of the curve. This model takes into account the background signal in the assay, which becomes more important if the dynamic range is low. The models described by eqs. 1 and 2 will result in similar values if there is a large magnitude in difference between E_{min} and E_{max} (~≥10-fold). The γ or k values have potential significance in predictions of clinical DDI; however, its use in current DDI prediction models has not been reported.

In comparing PXR transactivation data with actual clinical DDI effects, Persson et al. (2006) used several approaches to rank order clinical DDI potential with respect to three categories: inducers, weak inducers, or noninducers in vivo (or lack of evidence for induction in the literature). Some of the models used were based upon EC_{50} only, E_{max}/EC_{50}, or C_{max}/EC_{50} values. Although the data were not presented in this publication, it was discussed that these three models did

not successfully group the 25 compounds examined according to known CYP3A induction properties in vivo. When compounds were ranked based upon AUC_{total}/EC_{50}, the CYP3A in vivo inducers were grouped together with the exception of primidone. Ranking the compounds by $AUC_{unbound}/EC_{50}$ clustered all in vivo inducers, except primidone and troglitazone. After taking the extent of the induction response (eq. 3) into consideration, the classification of compounds did not improve.

Sinz et al. (2006) categorized PXR transactivation data from 170 drugs and natural products with high, moderate, or low clinical induction potential based upon the percentage of transactivation response of the compound (relative to the percentage of maximal RIF response) at the efficacious $C_{max,total}$. For compounds where the percentage of transactivation relative to rifampicin reached a plateau, an EC_{50} was calculated from the curves. For all other compounds, the following parameters were reported: 1) maximum percentage of transactivation and 2) the concentration at which maximum transactivation was measured. The criteria for clinical DDI were defined with the following values: >40% for high potential; between 15 and 40% for moderate potential; and <15% for low potential. Limited solubility and/or cytotoxicity may confound the application of these criteria for some compounds. Overall, the approach was found to be useful because it identified all of the potent known inducers and several moderate inducers with no false-positive compounds in the high-risk category.

The incorporation of clinical drug concentrations of the inducer (e.g., C_{max} or AUC) in addition to the transactivation response parameters is essential to avoid the prediction of false positives. For example, based on transactivation data, simvastatin was ranked as having a high induction propensity based on the E_{max}/EC_{50} ratio (El-Sankary et al., 2001), yet simvastatin does not induce CYP3A4 at clinically relevant exposures (Prueksaritanont et al., 2000). Therefore, the magnitude of hPXR activation needs to be viewed in light of the drug concentrations that will be needed in the clinic for target engagement. Overall, PXR data are useful for categorizing DDI potential of compounds with respect to CYP3A induction. It is amenable to screening in early drug discovery to identify compounds causing induction via activation of PXR, because it is used by the majority of PhRMA companies surveyed.

A*h*R Assays. In the unliganded, inactive state, A*h*R resides in the cytoplasm, in complex with two molecules of the chaperone protein heat shock protein 90, the immunophilin-like X-associated protein-2, and a 23 kDa cochaperone protein referred to as p23 (for reviews, see Kawajiri and Fujii-Kuriyama, 2007; Beischlag et al., 2008). Upon ligand binding, the A*h*R is believed to undergo a conformational change that exposes a nuclear localization sequence resulting in translocation of the complex into the nucleus. Release of the ligand-A*h*R from this complex and its subsequent dimerization with an A*h* nuclear translocator (Arnt) protein converts the A*h*R into its high-affinity DNA binding form. By binding the complex to its specific DNA recognition sites, the xenobiotic response elements, upstream of CYP1A2 and other target genes, stimulates transcription of these genes. 2,3,7,8-Tetrachlorobenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC), and β-naphthoflavone (BNF) are known, strong A*h*R ligands. It is interesting to note that some compounds (e.g., omeprazole) have been shown to induce A*h*R target genes but do not appear to competitively bind to the A*h*R. The reason for this effect is not completely clear (Daujat et al., 1992).

Most companies (89%) consider activation of the A*h*R as somewhat important, but only 23% assess the induction potential of compounds in a reporter gene assay. Because HepG2 cells contain relatively high endogenous levels of the A*h*R, overexpression of the receptor by transfection is not required in these cells. Activation can be measured in a format similar to the PXR transactivation assay by transfecting cells with a reporter construct containing the xenobiotic response element linked to luciferase or another suitable reporter gene. Positive controls typically used in such experiments are TCDD, 3-MC, BNF, and the proton pump inhibitors omeprazole and lansoprazole.

Although activation of the A*h*R is viewed as an undesirable characteristic of drugs, a current challenge is to predict what EC_{50} or E_{max} value measured in vitro in relation to clinical concentrations of inducer will result in a clinically significant induction response as illustrated by the example of omeprazole. In cultured hepatocytes, omeprazole induces CYP1A2 mRNA, protein, and catalytic activity (Diaz et al., 1990), and the EC_{50} for activation of the A*h*R was 100 μM in a reporter assay (Quattrochi and Tukey, 1993). In a study conducted with cancer patients, omeprazole induced both CYP1A2 protein and catalytic activity significantly after a 4-day treatment period (Diaz et al., 1990). However, studies conducted in healthy subjects showed a weak or no reduction in the exposure of CYP1A2 substrates after administration of omeprazole at therapeutic dose (Xiaodong et al., 1994; Andersson et al., 1998; Ko et al., 1999). In studies done with patients who demonstrate a poor metabolizer phenotype for CYP2C19 substrates, omeprazole decreased the exposure of the CYP1A2 substrate caffeine. Because omeprazole clearance is largely determined by CYP2C19, omeprazole plasma concentrations are markedly higher in poor than in extensive metabolizers (C_{max} 554 ng/ml and 2782 ng/ml in extensive and poor metabolizers, respectively) (Uno et al., 2007). Although the plasma level of omeprazole in poor metabolizers is still markedly lower than the EC_{50} measured in an A*h*R transactivation assay, the example of omeprazole reinforces the importance of interpreting in vitro-derived induction data in the context of systemic exposure of a drug.

CAR Assays. The relevance of activation of the CARs was valued from "don't know" to "very important" by survey respondents. This result is probably a reflection of the fact that at present, no company has an assay available to monitor activation of CAR in vitro, and therefore the significance of CAR activation is difficult to assess on a routine basis. Whereas in unchallenged hepatocytes or in vivo, CAR is sequestered in the cytoplasm, and in continuously cultured cell lines it is localized in the nucleus where it is constitutively active, even in the absence of a ligand. In hepatocytes treated with CAR activators, CAR translocates to the nucleus by a complex, not completely understood mechanism (Kawamoto et al., 1999; for review, see Kodama and Negishi, 2006), where it activates transcription of certain P450s, UDP-glucuronosyl transferases, glutathione transferases, and other genes (Maglich et al., 2002; Ueda et al., 2002). The activation mechanism of CAR is different from PXR, because the latter has a low basal activity and is highly activated upon ligand binding. CAR response elements have been mapped in a number of human genes, including CYP2B6, CYP3A4, members of the CYP2C family, and UGT1A1 (Swales and Negishi, 2004). CITCO, artemisin, and phenobarbital are known activators of CAR. It is interesting to note that phenobarbital does not bind to CAR directly but stimulates its translocation to the nucleus by dephosphorylation of a serine residue (Kawamoto et al., 1999; Hosseinpour et al., 2006). Analysis of CAR is further complicated by the identification of a number of alternatively spliced transcripts, encoding variants with insertions or deletions in the ligand-binding domain (Auerbach et al., 2003). One of the variants, CAR3, demonstrated low basal activity and could be activated by a number of both direct and indirect CAR activators in a transactivation assay (Faucette et al., 2007). Although less promiscuous than PXR, based on the information available, activation of CAR can cause induction of a wide range of genes, but currently measuring

activation of this receptor is difficult due to the lack of availability of a bona fide transactivation assay. Faucette et al. (2007) have reported that CAR agonists produce stronger CYP2B6 induction compared with CYP3A4 induction.

Summary: Nuclear Receptor Assays. The relevance of the various nuclear receptors during drug discovery and development was rated differently by the various companies. Use of PXR activation assays to predict enzyme induction potential was considered by a majority of companies to be very important (83%), whereas CAR (54% somewhat important, 31% very important) and AhR (89% somewhat important, 11% very important) assays were generally considered to be somewhat important.

There are currently no regulatory requirements to measure activation of nuclear receptors in reporter assays, and pharmaceutical companies mostly use these assays to guide their discovery programs. Because reporter assays are relatively high throughput and cost effective, they can be a valuable tool in drug discovery. In addition, an understanding of the molecular mechanism by which a drug causes a clinically significant positive induction response is advantageous in that it can be used as a guide to predict which other enzymes or transporters could be induced other than for instance CYP3A4. This method is especially the case for PXR and A*h*R for which reliable transactivation assays are available. Due to the complexity of CAR-mediated gene regulation, in vitro data for this receptor are difficult to interpret at present. The use of receptor activation assays is not recommended as the sole guidance to assess the induction potential of NMEs, because it cannot be excluded that receptors other than the ones routinely assessed could cause induction or that metabolites not formed in immortalized cell lines could be inducers. In addition, it should be realized that cross-talk between the receptors causing enzyme induction with many signal transduction pathways has been reported, suggesting that xenobiotics may affect a broad range of biological processes (for review, see Pascussi et al., 2008).

Hepatocyte Assays. Cultured primary hepatocytes and immortalized hepatocytes are the two models used to evaluate in vitro induction of drug-metabolizing enzymes. The experimental conditions, advantages, and disadvantages of these systems are discussed below.

Primary Hepatocytes Assays. With appropriate culture conditions, human hepatocytes remain differentiated and retain the ability to respond to inducers for a period of time. Cultured, primary human hepatocytes are the most accepted (industry, academia, regulatory) in vitro system for assessing the potential for drug candidates to induce human P450 expression (Hewitt et al., 2007c; LeCluyse et al., 2005). Human hepatocytes are a cellular system comprised of human receptors, coactivators and repressors, target genes and promoters, as well as human drug-metabolizing enzymes capable of biotransforming drugs. These properties are analogous to the liver and are essential to effectively model the inducibility of drug candidates and their metabolites. More recently, our knowledge and understanding of species specificity of the mammalian P450 isoforms and their interactions with other drugs have increased significantly. The failure in using animal data to routinely predict enzyme induction in humans is well documented (Kocarek et al., 1995); hence, the need for a human-relevant model system becomes more apparent. Primary human hepatocyte culture systems have been shown to effectively model human in vivo induction responses and are recognized as an effective tool for assessing induction potential (Hewitt et al., 2007c). The enzyme induction data from in vitro methods are known to correlate well with clinical observations, provided the in vitro experiments are performed at pharmacologically relevant concentrations of drug (LeCluyse et al., 2000). When hepatocytes are cultured using appropriate conditions that facilitate liver-like cell morphology and expression of liver-specific proteins, P450 enzymes are effectively induced in vitro analogous to the in vivo situation in terms of the magnitude and specificity of induction (LeCluyse et al., 2000; Runge et al., 2000). In addition to P450 enzymes, numerous studies have been reported using primary hepatocyte culture systems to assess induction of a variety of gene targets, such as Phase II enzymes and transporters (Raucy et al., 2002, 2004; Raucy, 2003; Kodama et al., 2004).

Although fresh human hepatocytes are the standard for evaluating in vitro induction of P450 enzymes, attachable cryopreserved hepatocytes have also been used. The drug-metabolizing enzymes remain inducible after cryopreservation, and due to the significant variation in activities of drug-metabolizing enzymes between individual human livers, certain lots of cryopreserved cells can generate results essentially indistinguishable from freshly isolated cells (Scherer et al., 2000; Hewitt et al., 2007c). Catalytic activities, mRNA, and protein expression of CYP1A2, 2B6, 2C9, 2E1, and 3A4 in cryopreserved hepatocytes have been shown to be inducible by standard P450 inducers (Roymans et al., 2005). The advantage that cryopreserved cells offer over fresh isolates is that experiments can be planned in advance and are not dependent on the sporadic availability of fresh primary hepatocytes.

In general, a test compound is evaluated in hepatocyte cultures from several different donors, in addition to a vehicle control and a positive control for 2 to 3 days, with medium changed every 24 h during incubation. Test compounds are evaluated at or around anticipated therapeutic concentrations, and the positive control incubation is used to assess the suitability of each donor preparation and often as a comparator to induction observed by the test compound. Enzyme induction potential can be evaluated by measuring increases in enzyme activities, mRNA levels, or protein expression (Western immunoblotting). The data from cultured hepatocyte experiments can take several forms, such as -fold or percentage increase compared with the vehicle control, percentage increase compared with the positive control, or EC_{50} determination based on data generated from enzyme activity or mRNA expression.

The survey indicates that nearly all companies use primary human hepatocytes to characterize the enzyme induction potential of compounds in drug development, and the majority of companies also use them to evaluate enzyme induction in drug discovery. As noted in the past, the major limitation to the use of primary human hepatocytes continues to be cost and availability. Many companies conduct hepatocyte experiments in-house with cells purchased from commercial vendors; moreover, most companies indicated that outsourcing to contract research organizations was commonly used in combination with in-house experiments. A few companies indicated that they isolated human hepatocytes in-house or obtained cells from hospitals or universities.

Researchers indicated that they are comfortable using both freshly isolated hepatocytes and cryopreserved-attachable hepatocytes because it has been shown that induction responses are similar between fresh hepatocytes and attachable lots of cryopreserved hepatocytes (Roymans et al., 2005; Hewitt et al., 2007c). Respondents did indicate that in drug discovery, cryopreserved cells were used more often due to increased availability, whereas combinations of fresh and cryopreserved cells were used in drug development. Furthermore, three donors were routinely evaluated when assessing enzyme induction of compounds in development, whereas only one donor was typically used in drug discovery. Donor characteristics that should be avoided because they were considered undesirable and could affect the outcome of the experiment or viability of the cells included the following: a high body mass index or fatty livers; liver disease, such as viral infections; and very young or elderly donors (<6 months or >60

years of age). The most common culture format was equally distributed between collagen monolayer and collagen-Matrigel sandwich culture. Depending on the endpoint (RNA expression or enzyme activity) the plate formats varied slightly, although the most common format for both applications was the 24-well plate. When only RNA expression was considered, 24- and 96-well plates were the most common, whereas when only enzyme activity was measured, 24-, 48-, and 96-well formats were most commonly used. However, when enzyme activity was to be measured from microsomes isolated from cultured hepatocytes, Petri dishes or 6-well plates were often used. Once plated, the cells were generally allowed 1 to 2 days to recover or adapt to the cell culture conditions.

A variety of cell culture media were described in the survey responses [William's E, InVitroGRO (Celsis, Chicago, IL), Dulbecco's modified Eagle's medium, hepatocyte maintenance, Chee's, HepatoSTIM (BD Biosciences, Franklin Lakes, NJ)], yet the most common were William's E and InVitroGRO HI. Regarding media supplements, common additions included ITS (insulin, transferrin, selenium), dexamethasone, and antibiotics (penicillin-streptomycin), similar to what is commonly reported in the literature (LeCluyse et al., 2005). Other less commonly described media additions included fetal bovine serum, linoleic acid, and antifungals. In some instances, there was mention of different media/supplements for plating and culturing cells. Most common was the addition of serum to the media after plating, which was then removed, and the experiment continued with serum-free media. Test compounds or positive controls were typically dissolved in dimethyl sulfoxide (DMSO) and incubated at a final concentration of 0.1% DMSO. Other solvents, such as acetonitrile, methanol, ethanol, and higher concentrations of DMSO were noted as alternative dissolution methods; however, they were typically used only when 0.1% DMSO was not applicable. The minimum number of testing concentrations was three but ranged up to 10, and duplicate or triplicate replicates were generally used.

The enzyme most routinely evaluated was CYP3A4, whereas enzymes such as CYP1A2, CYP2B6, and CYP2C9 were also often evaluated. Other enzymes and transporters such as CYP2C8, CYP2C19, UGT1A1, and MDR-1 were occasionally evaluated. The most frequently used positive controls were as follows: CYP1A2 (omeprazole, 20–50 μM); CYP2B6 (phenobarbital, 750-1000 μM); and CYP2C9 and CYP3A4 (rifampicin, 10–25 μM). Other less common positive controls were BNF (10–30 μM) for CYP1A2 and CITCO (2 μM) or rifampicin (10 μM) for CYP2B6. The length of cell treatment with positive controls and test compounds overlapped between RNA expression and enzyme activity measures but were generally 2- and 3-day treatments, respectively. To have confidence in the data generated from test compounds, researchers have adopted various levels of acceptance criteria for donor preparations based on the RNA or activity response of positive controls. In general, 2- to 10-fold increases in RNA expression or 2- to 4-fold increases in enzyme activity were noted as acceptable donor responses to positive controls; however, the minimum of 2-fold was the most common response by far for enzyme activity.

The most common endpoint for assessing enzyme induction was measuring enzyme activity (100% of respondents) with the majority of respondents also measuring RNA expression (77%). When measuring enzyme activity, the single probe substrate incubated in situ was the most common method with a much smaller percentage of researchers using cassette probes in situ. The second most common method of measuring enzyme activity was by means of microsomal incubations (i.e., microsomes isolated from plated hepatocytes). With regard to measuring RNA expression, the most common method was reverse transcription-polymerase chain reaction followed by several other methods, such as branched DNA, nuclease protection, Invader (Third Wave Technologies, Madison, WI), and Affymetrix (Santa Clara, CA). Both RNA expression and enzyme activity data require normalization to an endogenous component or cell number. In the case of mRNA expression, housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase, 18S, or actin are recommended. For normalization of the catalytic activity, cell number, total protein, or DNA content can be used.

Immortalized Hepatocytes Assays. Primary human hepatocytes are commonly used in enzyme induction studies; however, their limited supply and significant donor-to-donor variation complicate their application in early drug discovery. Consequently, there is a need to generate human hepatocyte-like cells that provide a continuous supply while maintaining stable expression of necessary enzymes, transporters, and nuclear hormone receptors for routine screening and characterization of enzyme induction. Immortalized hepatocytes are one example of cells that can grow and divide indefinitely under optimal culture conditions and can occur naturally (e.g., tumorigenic cells isolated from hepatocarcinomas) or by converting primary hepatocytes into nontumorigenic immortalized cells (Sinz and Kim, 2006).

Several lines of hepatocarcinoma cells (e.g., HepG2, BC2, HepaRG) have been evaluated for their ability to mimic the enzyme induction response of primary human hepatocytes. Among them, the HepG2 cell line has been well characterized and is widely used throughout drug metabolism and toxicology testing. HepG2 cells have demonstrated robust induction response to CYP1A inducers; however, due to the low basal expression of enzymes and little to no induction response to known CYP3A4 inducers, HepG2 cells are not considered an appropriate model to study drug-drug interactions (Westerink and Schoonen, 2007; Harmsen et al., 2008). Gómez-Lechón et al. (2001) performed an extensive analysis of the biotransformation properties of BC2 cells by measuring basal and inducible expression of Phase I and II enzymes. Their results showed an increase in CYP1A1/2 enzyme activity (8-fold) by methylcholanthrene, CYP2B6 activity (1.7-fold) by phenobarbital, and CYP3A4 activity (5-fold) by dexamethasone. HepaRG cells, when cultured to confluency for several weeks under specific culture conditions, develop into a highly differentiated hepatocyte-like cell line (Gripon et al., 2002). A comprehensive expression analysis performed by Aninat et al. (2006) showed that RNA expression of major transcription factors (PXR, AhR) were similar between primary human hepatocytes and differentiated HepaRG cells, and the expression of CAR was 20 to 30% of that found in human hepatocytes. Kanebratt and Andersson (2008) have demonstrated increases in mRNA and activity using the HepaRG cell line and prototypical inducers. However, the expression of enzymes and nuclear receptors as well as the response to enzyme inducers have been shown to vary depending on the media composition and culture conditions (Aninat et al., 2006).

The Fa2N-4 cell line is derived through immortalization of primary human hepatocytes using the SV-40 large T antigen. Using known inducers of P450 enzymes, Mills et al. (2004) reported concentration-dependent increases in both transcript levels and enzyme activities of CYP3A4, CYP2C9, and CYP1A2. These changes were comparable with induction responses observed in primary human hepatocytes. In addition, UGT1A and MDR-1 were also induced by rifampicin treatment in this cell line. Unfortunately, Fa2N-4 cells have very low expression of CAR and several drug transporters; therefore, CAR-mediated induction of CYP2B6 cannot be evaluated, the induction of CYP3A4 may be attenuated, and cellular uptake of drugs may be reduced or altered (Hariparsad et al., 2008).

LS180 is a human colon carcinoma cell line routinely used and well characterized in studying intestinal absorption of drugs and regulation

of CYP3A4 and MDR-1. In LS180 cells, CYP3A4 and MDR-1 have been shown to be responsive to induction stimuli by reserpine, rifampicin, phenobarbital, and verapamil, as well as induction of CYP1A2 when exposed to the prototypical CYP1A inducer, omeprazole (Schuetz et al., 1996; Brandin et al., 2007). Overall, LS180 cells seem to be a suitable model to study the regulation of CYP3A4 and MDR-1 in the intestine, although there is evidence to suggest that this cell line may not recapitulate the induction response in liver tissue or hepatocytes due to altered levels of the nuclear receptor corepressor (Zhou et al., 2004).

The survey results indicate that a majority of companies do not use immortalized hepatocytes for routine screening; however, ~30% of companies use the Fa2N-4 cell line in drug discovery to assess CYP1A and CYP3A4 enzyme induction. As an example, Ripp et al. (2006) developed a higher-throughput induction assay using Fa2N-4 cells in 96-well plates and produced detailed concentration-response curves for known inducers of CYP3A4. Although the Fa2N-4 cell line has the ability to evaluate induction of other enzymes, as well as transporters, none of the companies indicated evaluation of induction beyond CYP1A and CYP3A4. The most common experimental conditions were 3-day treatment of cells with approximately 5 concentrations of test compound (in triplicate) in a 24-well plate format. The common positive controls used were rifampicin (10 μM) and omeprazole (10 μM). Enzyme activity using probe substrates and RNA expression by reverse transcription-polymerase chain reaction were the common endpoints measured in each assay. Interpretation of results was generally characterized by percentage of activity or RNA expression compared with the positive control.

In summary, immortalized hepatocytes seem to be useful in evaluating well characterized induction mechanisms (e.g., PXR- or AhR-mediated). However, no one cell line affords an exact reproduction of a hepatocyte, and most companies felt that these cell lines are not fully understood or characterized. For example, unknown expression of nuclear receptors or transcription factors as well as drug transporters and cytotoxic effects were noted as variables that are not fully understood across cell lines. Therefore, due to these limitations, we do not consider that immortalized hepatocyte cell lines are an adequate replacement for primary human hepatocytes; however, they may be used to complement the use of primary human hepatocytes, especially for higher-throughput applications in drug discovery and early stages of drug development.

Hepatocyte Assays: Interpretation of Data. Interpretation of hepatocyte induction data are a highly debated topic with respect to predicting clinical outcome even though several methods exist for assessing the drug-drug interaction potential of enzyme inducers. All of the respondents indicated they used the "percent of positive control" in the interpretation of enzyme induction data, and nearly half of the respondents also used "fold induction above vehicle control."

The "40% of positive control" criteria, originating initially from the PhRMA position article (Bjornsson et al., 2003), was set to establish a true positive induction signal in in vitro assays. The value was set particularly for activity data because, in general, the dynamic range of mRNA induction is greater. In the case where the maximal induction levels of the positive control are only 4- to 5-fold (in the range of acceptability), anything less than 40% of this would be in the background of the assay. In some cases where positive control inducers (e.g., for CYP1A2) elicit a very high induction response, discretion must be taken as to whether the 40% cut-off criteria is appropriate for induction of P450 activity in vitro. From the current survey, it is clear that better predictive models are needed to aid in understanding the correlations between in vitro data and in vivo effect with respect to induction. In particular, those models that relate the in vitro induction response to clinically relevant exposures, either as a correlation (described later) or more physiologically based models, which take into account the pharmacokinetics of the inducer and affected substrate, transporters, and mixed mechanisms (P450 reversible and time-dependent inhibition and induction), would be of the most value. In addition to the percent of positive control, 15% of the respondents also determined EC_{50} values in this assay (be it mRNA, activity, or protein level; the endpoint assay was not discerned). However, from the comments made, most companies are relating the concentration-dependent induction response to known or expected clinical exposures.

When assessing percentage of positive control or other ratio methods that incorporate therapeutic drug concentrations, most often the C_{max} total drug concentration was used (71%), although some researchers indicated the use of unbound C_{max} drug concentration (36%). Even though the unbound C_{max} drug concentration conforms to the "free drug hypothesis" and theoretically may be more accurate, the more conservative approach of using total C_{max} drug concentration appears to be predominant when assessing enzyme induction potential. The section on in vitro-in vivo correlations (IVIVC) contains further discussions on data analysis and interpretation. Finally, the actual therapeutic drug concentration may not be known with a great deal of confidence during preclinical testing or early human clinical trials; therefore, it is common to evaluate enzyme induction over a range of drug concentrations. The survey indicates that most researchers would consider enzyme induction as being significant when increases in enzyme activity are observed within 2- to 5-fold of the anticipated C_{max} at therapeutic dose.

Several common situations were noted in the survey responses when interpreting enzyme induction data. They include the following: 1) increases in RNA or enzyme activity at low drug concentrations with decreases in RNA or activity at higher concentrations (inverted U-shaped response curve); 2) increases in RNA expression with little or no increase in enzyme activity; and 3) an enzyme induction response is observed in only one of several donor preparations. The first situation (inverted U-shaped response) is typical of compounds that exhibit poor solubility in culture media or cytotoxicity. Although drug dissolution in the original neat DMSO solution is readily discernable, the survey indicates that only 46% of researchers confirmed the solubility of test compounds in culture media. However, most survey respondents indicated that cytotoxicity was commonly assessed during the course of an experiment. The most common methods for assessing cytotoxicity were cell morphology, enzyme leakage, ATP content, or mitochondrial function (MTT). Even though solubility and/or cytotoxicity are often used to explain inverted U-shaped dose-response curves, there have been several cases reported for which solubility or toxicity were not the apparent cause (Raucy, 2003; Ripp et al., 2006). Therefore, the inverted U-shaped dose-response curve may in some cases represent a true underlying pharmacology that is incompletely understood at this time. In the second case of increased RNA expression with little to no increase in enzyme activity, this example is common when the test compound is both an inducer and inhibitor of the same enzyme. The RNA expression is unaffected by the enzyme inhibition properties; however, the enzyme activity measurements can be confounded by concomitant inhibition and induction. This situation is commonly encountered when enzyme activity is measured in situ and the test compound is not adequately removed from the hepatocyte culture; however, this situation can often be overcome by measuring enzyme activity with isolated microsomes. In addition, removing the drug from the culture system will eliminate the inhibition properties of reversible inhibitors, but often the test compounds are both reversible and irreversible inhibitors (mechanism-based or time-dependent inhibitors). In this case, the

enzyme becomes inactivated or nonfunctional due to the affects of the irreversible inhibitors. The interpretation of this phenomenon is more difficult and complex; the drug is clearly an inducer (based on RNA expression information); however, the prediction of anticipated drug-drug interactions will be affected by the simultaneous induction-inhibition properties of the test compound. The most well known drug that exhibits these properties is ritonavir, which predominantly presents as an inhibitor of CYP3A4, yet in some patients, evidence for drug interactions due to enzyme induction have been reported (Hsu et al., 1998). Due to this interplay between inhibition and induction of P450 enzymes, we recommend using both enzyme activity and RNA expression endpoints to fully understand and interpret enzyme induction in cultures of primary human hepatocytes. Ultimately, the drug-drug interaction (pharmacokinetic changes), if needed, should be evaluated in well designed clinical drug interaction studies, because the in vitro data will be difficult to extrapolate to in vivo effects. Finally, when only one of several donor hepatocyte preparations indicates a positive induction response, the result cannot be ignored or discounted as an outlier. However, this type of result will typically elicit additional mechanistic studies or repeat hepatocyte studies to better understand the spurious nature of the positive result.

Hepatocyte Assays: Recommendations. The most commonly used and recommended experimental protocol for assessing enzyme induction in primary human hepatocytes for regulatory submissions is as follows.

- Fresh or platable cryopreserved hepatocytes, as either monolayer or sandwich culture, with a 1 to 2 day recovery period after plating.
- Treatment with NME and positive controls for 2 to 3 days (changing medium with test compounds every 24 h) in media containing ITS, dexamethasone, and penicillin-streptomycin as media supplements.
- Test compounds dissolved preferably in DMSO (v/v 0.1%) whenever possible and incubated at three or more different concentrations (in triplicate), spanning anticipated or known therapeutic concentration range including a concentration at least an order of magnitude higher. Alternate solvents and NME concentration ranges may be used, as appropriate.
- The recommended positive controls include omeprazole (25–50 μM), phenobarbital (1000 μM), and rifampicin (10 μM) for CYP1A2, 2B6, and 3A4, respectively, at concentrations known to elicit maximal induction response.
- Assessment of cytotoxicity of the NME under the experimental conditions.
- Knowledge of aqueous solubility characteristics and visual assessment of solubility in the culture medium at the concentrations tested.
- Catalytic activity and mRNA assessments for CYP1A2, CYP2B6, and CYP3A4.
- For a hepatocyte induction experiment to be acceptable, CYP1A2, CYP2B6, and CYP3A4 positive controls should exhibit ≥2-fold vehicle control catalytic activity and ≥6-fold vehicle control mRNA level.
- At least three donor hepatocytes, with experiment meeting the acceptable criteria for each donor.
- A positive result in at least one of the three donor hepatocytes is considered an indication of induction.
- Interpretation of results are conducted by an empirical approach such as percentage of change compared with the positive control or a mathematical or correlation-based approach, using the therapeutic C_{max} drug concentration at steady state as a benchmark. The use of free (unbound) or total (bound and unbound) drug concentrations for data interpretation may be based on the available historical data and/or models used at each laboratory.

In Vitro-in Vivo Correlations. Some common approaches to data interpretation and IVIVC have been briefly discussed in the previous sections on transactivation and hepatocyte assays. Because IVIVC is a topic of critical importance, a more in-depth analysis is presented below. This discussion is based on the review of the current literature, implicit information from the survey, and analyses by the authors, and is not based on specific survey results.

It can be appreciated that whatever in vitro endpoint was used to assess induction potential (e.g., nuclear receptor transactivation, mRNA, or enzyme activity from human hepatocytes), the magnitude of the maximal in vitro induction effect (E_{max}), in vitro induction potency (EC_{50}), character of the interaction (e.g., full or partial agonists, etc.), and an idea about actual clinical exposure are all needed to determine whether a compound will probably be an inducer of P450 enzymes in humans in vivo (Smith et al., 2007). The number of different published models to predict the clinical outcome of P450 induction are limited; however, the numbers of reports, particularly of modeling CYP3A induction in vivo, have increased very recently. Those models that have been reported are based upon the same concept, that being the law of mass action for receptor binding (Kato et al., 2005; Ripp et al., 2006; Fahmi et al., 2008a,b; Kanebratt and Andersson, 2008; Shou et al., 2008). The E_{max} model (eq. 4) is generally used to describe an induction effect based upon EC_{50} and E_{max} values obtained in in vitro assays in relation to an inducer concentration ([Ind]), i.e., a therapeutic effective in vivo concentration.

$$\text{Effect} = \frac{E_{max} \times [\text{Ind}]}{EC_{50} + [\text{Ind}]} \quad (4)$$

The endpoints measured in primary (or immortalized) human hepatocytes are typically CYP3A mRNA and/or enzymatic activity. With regard to what in vitro endpoint is more amendable to predictions of clinical outcomes, mRNA data are most often used and are considered appropriate based upon several factors: 1) the induction process involves receptor binding and transactivation of the gene transcription, and hence mRNA production is a more direct measure of this event than enzyme activity; 2) measurement of mRNA provides a facile measure with better dynamic range than enzyme activity measurements; and 3) measurement of P450 activity in situ, which is the most common means for assessing enzyme activity, can potentially result in false negatives in the case of time-dependent or mechanism-based inhibitors, or potent reversible inhibitors with high metabolic stability in hepatocyte cultures. To this point, a good correlation between CYP3A4 mRNA and activity increases with inducers has been found in human hepatocytes when time-dependent inhibitors (mechanism-based inhibitors or formation of metabolites that are potent reversible inhibitors) are excluded (Fahmi et al., 2008a).

PXR Transactivation Data. As described in a previous section, PXR transactivation assays have been used to generally rank order (El-Sankary et al., 2001) or qualitatively categorize the in vivo potency of CYP3A inducers (e.g., high, moderate, low DDI potential) with respect to known clinical DDI (Persson et al., 2006; Sinz et al., 2006). Direct extrapolation of PXR activation data for quantitative prediction of DDI magnitude in vivo has not been reported at this time. It remains to be determined whether it has quantitative DDI prediction potential with respect to CYP3A induction, for instance, because it may have its limitations due to the induction of CYP3A by other mechanisms, e.g., CAR.

Human Hepatocyte Induction Data. Modeling approaches to predict clinical DDI magnitude of P450 inducers using in vitro data have only been reported recently. The models have been almost exclusively for predictions of CYP3A induction but may be amendable to the induction of other P450s. The relatively limited reports of clinical induction with respect to other P450 enzymes are probably the reason that most models have been validated against CYP3A. In

general, there have been two types of approaches: mathematical prediction approaches and correlation (calibration curve) approaches, both of which are described below.

Mathematical prediction approaches. Prediction models have been reported that incorporate in vitro induction data expressed in the sigmoidal E_{max} model (eq. 1) or E_{max} model (eq. 4) into mathematical equations to predict changes in AUC of CYP3A substrates based upon the assumptions of the "well stirred liver" model for hepatic blood clearance. The first report to quantitatively predict clinical DDI magnitude with respect to P450 induction using in vitro induction data in the E_{max} model was by Kato et al. (2005). More recent reports have incorporated the fraction of the substrate metabolized by the affected P450 enzyme (f_{mCYP}) into the prediction model, because the magnitude of the DDI is dependent upon this value in addition to the properties of the inducer (Fahmi et al., 2008b; Shou et al., 2008). The more recent equations used to predict change in AUC of an orally administered affected CYP3A substrate by a CYP3A inducer are shown below in eq. 5 (Shou et al., 2008) and in eq. 6 (from Fahmi et al., 2008b, showing only the induction model). Parameters incorporated into the DDI predictions from these reports include the following: EC_{50} and E_{max} of CYP3A4 induction in hepatocytes (mRNA and/or activity); relevant clinical in vivo concentrations of inducer either as unbound or total ([Ind]); fractions of the victim drugs cleared by CYP3A (f_{mCYP}); and, in Shou et al. (2008), the fraction unbound of the inducer in hepatocytes (fu_{hepr}). Fahmi et al. (2008b) also incorporated a model to account for intestinal availability of CYP3A substrates in the presence and absence of a CYP3A inducer, as well as models to incorporate concurrent P450 inhibition or inactivation (data not shown). The model in eq. 5 was used to predict the actual clinical DDI of six inducers in 103 clinical DDI trials from in vitro induction data (enzyme activity, two hepatocyte donors), and it was found that the best correlations ($r^2 = 0.578–0.624$) of the modeled outcome to actual clinical DDI were found when using unbound [Ind] and fu_{hepr}. The predictability of eq. 6 was dependent upon the value "d," a parameter needed for in vitro-to-in vivo scaling of the induction data (mRNA), which was optimized by linear least-squares regression that maximized the accuracy of the global data set. The overprediction of DDI with RIF using mRNA data by Shou et al. (2008) has been speculated to be due to the much greater E_{max} values for induction of mRNA compared with enzyme activity. The need for a scalar or normalization to a positive control maximal E_{max} value is apparent, and mRNA data may be more affected because the dynamic range is greater than that of enzyme activity. Normalizations of E_{max} should be performed to predict clinical DDI, due to the large interindividual variability in maximal P450 induction in vitro and to scale the in vitro effect to the in vivo clinical effect.

$$\frac{AUC_{po,i}}{AUC_{po}} = \frac{CL_{int}}{CL_{int,i}} = \frac{1}{f_{m,CYP} \times \left(1 + \frac{E_{max} \times [Ind]^n}{(EC_{50} \times fu_{hept})^n + [Ind]^n}\right) + (1 - f_{m,CYP})} \quad (5)$$

$$\frac{AUC_{po,i}}{AUC_{po}} = \frac{CL_{int}}{CL_{int,i}} = \frac{1}{f_{m,CYP} \times \left(1 + \frac{d \times E_{max} \times [Ind]}{(EC_{50}) + [Ind]}\right) + (1 - f_{m,CYP})} \quad (6)$$

In addition to the mathematical models, described above, the physiologically based drug interaction model, Simcyp Population-Based ADME Simulator (Simcyp Ltd., Sheffield, UK), has recently incorporated a model to predict clinical induction from in vitro induction parameters (Rostami-Hodjegan, 2009). This model accounts for normalization of the E_{max} value by the maximal positive control response, as well as concurrent P450 inhibition and inactivation.

Correlation (calibration curve) prediction approaches. There has been success with more empirical approaches to predict clinical DDI with respect to CYP3A induction from in vitro human hepatocyte data. Induction studies using immortalized cells (Fa2N-4 and HepaRG cells) and cryopreserved human hepatocytes have been used to correlate in vitro CYP3A4 mRNA induction results [as a "Relative Induction Score" (RIS) or AUC/F_2 value, described in more detail below] with the actual magnitude of in vivo CYP3A induction (Ripp et al., 2006; Fahmi et al., 2008a; Kanebratt and Andersson, 2008). These more empirical approaches require calibration curves with positive and negative controls. This calibration curve becomes very important when using different hepatocyte donors or to account for interexperimental variability when using immortalized cells or primary hepatocytes. It may be established that the EC_{50}s do not vary greatly, and it is probable that running positive and negative controls at the concentration at E_{max} would suffice in populating a curve. In the immortalized cell line (Fa2N-4 cells), the EC_{50} and E_{max} values varied with a coefficient of variation of 28 and 37%, respectively (Ripp et al., 2006). However, these values are likely to vary more with different primary hepatocyte donors. It is well recognized that the E_{max} value can vary considerably with donor. It is also likely that differences in cell membrane or protein binding, expression levels of nuclear receptors (e.g., PXR and CAR), coactivators, repressor proteins, and active drug transporters of different hepatocyte donors may also influence EC_{50} values. It is recommended to run full calibration curves to obtain EC_{50} and E_{max} until it is established that EC_{50}s do not vary considerably in the particular cell line or a particular cryopreserved hepatocyte donor.

RIS approach. The use of the RIS approach is described in Ripp et al. (2006). In this study, the researchers used Fa2N-4 cells to assess induction of CYP3A4 mRNA with various inducers and used the E_{max} model (eq. 4) as the RIS. The RIS was plotted versus the actual clinical mean percentage decrease in AUC of the CYP3A4 probe substrate, midazolam, or ethinylestradiol from the particular study referenced in Ripp et al. (2006). Compounds that were both inducers and time-dependent inhibitors of CYP3A4 within a therapeutically relevant concentration range were deliberately excluded from the Ripp et al. (2006) dataset. The IVIVC correlation was excellent using $C_{max,u}$ in the model ($r^2 \geq 0.92$) for both midazolam and ethinylestradiol. If total C_{max} was used instead of free C_{max}, the correlation dropped ($r^2 < 0.55$). In a recent study, the RIS approach was also evaluated by using human cryopreserved hepatocytes (Fahmi et al., 2008a). The correlation between RIS calculated using E_{max} and EC_{50} values from CYP3A4 mRNA induction data, and percentage decrease in midazolam or ethinylestradiol exposure was also excellent ($r^2 =$ 0.96 and 0.82, respectively). A benefit of the cryopreserved hepatocytes in this evaluation was a greater signal-to-noise ratio for induction, compared with the Fa2N-4 cells. In addition, Fa2N-4 cells do not express CAR and have altered expression of some hepatic transporters, which may effect intracellular inducer concentrations.

AUC/F_2 approach. The AUC/F_2 approach, as described by Kanebratt and Andersson (2008), was also successful in correlating an in vitro induction response to the actual clinical DDI magnitude of CYP3A substrates. In this method, F_2 was defined as the concentration of inducer leading to a 2-fold increase of the baseline levels of CYP3A4 mRNA in the hepatocytes (in this study, HepaRG cells were used). CYP3A4 mRNA induction data were fit to a Hill equation for

one-site dose response (eq. 2) to obtain the F_2 value. The F_2 value may also be obtained by visual inspection of the data where the 2-fold increase is observed. This approach may be of benefit when an E_{max} value cannot be reached, such as in the case of compounds with low solubility and/or cell toxicity.

In relation to exposure (AUC), the AUC/F_2 value was used to rank highest to lowest DDI potential. The correlation between in vitro and in vivo induction in this study was assessed by plotting AUC/F_2 versus the clinical percentage decrease in the AUC of a CYP3A probe substrate. The data were fit to an equation, analogous to the E_{max} model (eq. 4):

$$\text{Effect} = \frac{\%\text{decrease of in vivo AUC}_{max} \times (\text{AUC}/F_2)}{\%\text{decrease of in vivo AUC}_{50} + (\text{AUC}/F_2)} \quad (7)$$

A good correlation was reached when using AUC_{total} ($r^2 = 0.863$). A slightly lower correlation ($r^2 = 0.859$) was reached using unbound AUC values. When the maximum plasma concentration in vivo ($C_{max,total}$ and $C_{max,u}$) was used instead of AUC and AUC_u, the r^2 values were slightly lower (0.710 and 0.812, respectively). This approach seems to be promising, but it will need further assessment with data from primary human hepatocytes.

Comparisons of Correlation (Calibration Curve) Approaches to Predict Clinical CYP3A Induction. In an effort to compare different correlation approaches, it was desirable to evaluate these different models using a single dataset. Therefore, we have used the data from Ripp et al. (2006) to compare different correlation methods to correlate in vitro induction response data of CYP3A4 mRNA to actual in vivo clinical DDI with the CYP3A probe substrate, midazolam. The calibration approaches that were compared were the E_{max} model (eq. 4) (or RIS) as described in Ripp et al. (2006), C_{max}/EC_{50} (used previously for rank-ordering PXR transactivation data) (Persson et al., 2006), AUC/F_2 model as described in Kanebratt and Andersson (2008), and the E_{max} model multiplied by the ratio of NOEL (no observed effect level) to C_{max}. More recently, an addition to the E_{max}-based IVIVC model has been suggested (Hewitt et al., 2007a,b). Hewitt et al. (2007a,b) proposed that the highest concentration at which no induction response is observed (NOEL) in vitro should be incorporated in predictions of induction risk; however, no IVIVC using this model has been published (Hewitt et al., 2007b). The E_{max}, EC_{50}, and free and total C_{max} values of the compounds evaluated were as described by Ripp et al. (2006). The AUC values used in the AUC/F_2 model were as described by Kanebratt and Andersson (2008), and the AUC values were 8.3 and 27 μM/h for rosiglitazone (8 mg) and pioglitazone (45 mg), respectively (Malinowski and Bolesta, 2000; Christensen et al., 2005). The AUC values of compound A (40 and 120 mg) were 0.21 and 0.88 μM/h, respectively. The F_2 values were calculated for this data set as described by Kanebratt and Andersson (2008) or by visual inspection of the concentration versus induction effect data.

As reported previously (Ripp et al., 2006), there was an excellent correlation with predictions of clinical DDI using the E_{max} model (RIS) and $C_{max,u}$ ($r^2 = 0.927$) (Fig. 1A); however, using $C_{max,total}$, there was a poor correlation ($r^2 = 0.503$; data not shown). Using the same data set, the AUC/F_2 and C_{max}/EC_{50} correlation models using unbound $C_{max,u}$ also found excellent correlations with in vivo induction results ($r^2 = 0.932$ and 0.927) (Fig. 1, B and C). Poor correlations were found using $C_{max,total}$ in these models ($r^2 = 0.663$, AUC_{total}/F_2; $r^2 = 0.537$, $C_{max,total}/EC_{50}$, data not shown). Poor correlations were also found with incorporation of the NOEL/C_{max} in the E_{max} equation using total or unbound C_{max} concentrations ($r^2 = 0.644$ and 0.571, respectively; data not shown).

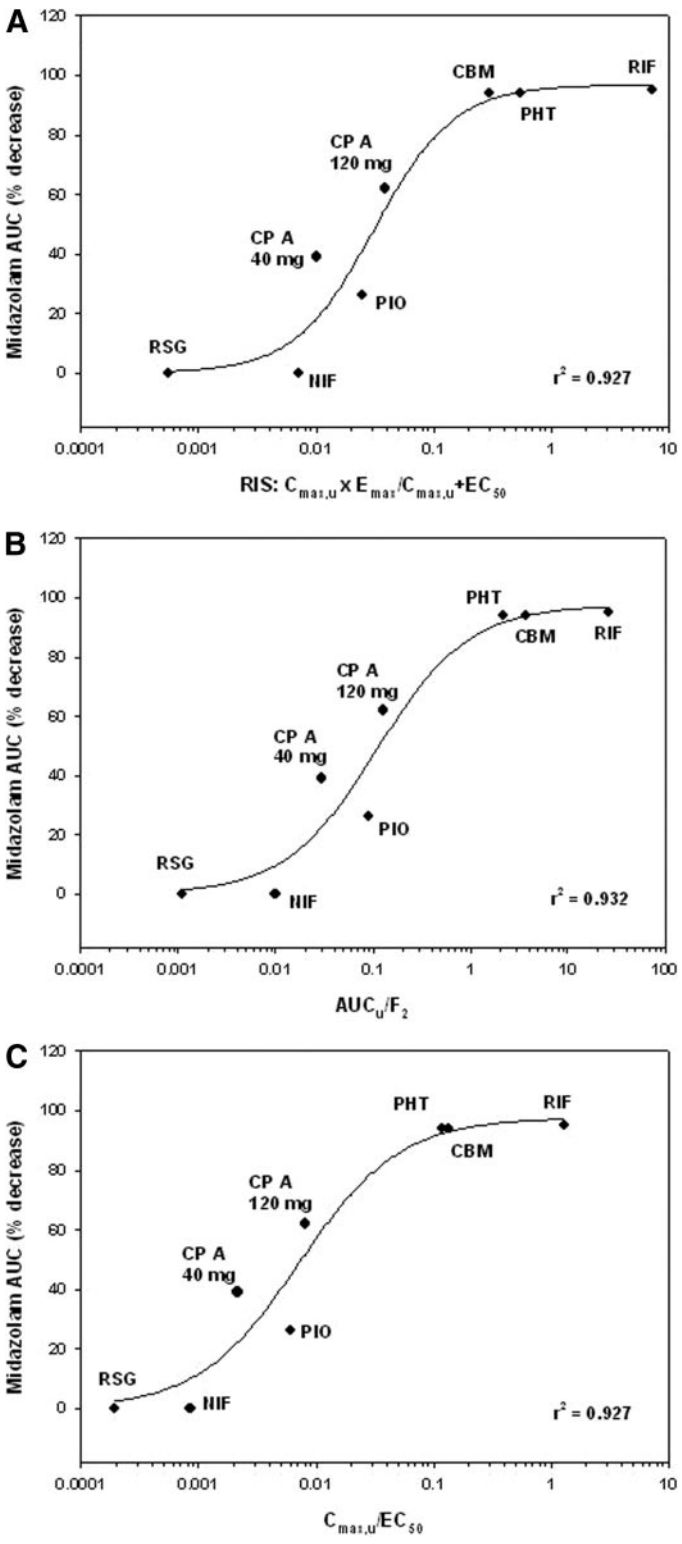

FIG. 1. Comparison of correlation (calibration curve) approaches to predict clinical induction of CYP3A. A, the RIS approach; B, the AUC/F_2 approach; and C, the $C_{max,u}/EC_{50}$ approach. Data are from Ripp et al., 2006. CBM, carbamazepine; CP A, 6-methoxy-1-methyl-trifluoromethyl-isochroman-7-ylmethyl)-(2-phenyl-piperidin-3-yl)-amine; NIF, nifedipine; PIO, pioglitazone; RSG, rosiglitazone; PHI, phenytoin.

Overall Recommendations for IVIVC Approaches and Future Directions. As discussed above, there are numerous mathematical and correlation approaches for establishing IVIVC for induction. Many of

these approaches have demonstrated utility in IVIVC, but they also have their disadvantages. Therefore, we are not recommending any single approach for use in all situations, but instead we recommend understanding the pros and cons of each approach and leave it to the investigator to decide on the best tool for a given situation. The correlation approaches are quite straightforward and amenable to predictions of clinical DDI with respect to CYP3A induction; however, they require an extensive amount of data to establish calibration curves. They may also not be appropriate in cases where compounds are both inhibitors and inducers of CYP3A. Mathematical model approaches, which have the capability to capture concurrent P450 inhibition, are probably more amenable for more comprehensive DDI predictions. The donor-to-donor or cell line variability in the maximal inducibility of the in vitro test systems (e.g., immortalized cell lines, primary hepatocytes) lead to the requirement for a calibrator (e.g., positive controls) in the mathematical model approaches to relate the in vitro response to the actual clinical response. Physiologically based models, although relatively new for the induction field, are promising tools for the future of in vitro to in vivo extrapolation of induction. In addition, most current IVIVC models were tested specifically for CYP3A; development of IVIVC models for induction of enzymes other than CYP3A is an area that requires more investigation.

Clinical Induction Studies. An NME may be a precipitant (inducer) and/or an object (substrate) of a pharmacokinetic drug-drug interaction due to enzyme induction. This article has focused on the assessment of NME as an inducer of P450 enzymes. In vitro induction studies with cultured human hepatocytes will provide guidance for the clinical induction studies to assess whether coadministration of the NME will result in decreased exposure of P450 substrates due to enzyme induction. The survey responses indicated that the company clinicians are very interested in knowing the in vitro data (72% very interested, 28% somewhat interested). The in vitro induction data are generally presented to the clinicians in the context of clinical concentrations, comparison to other therapeutics, clinical relevance, implications for labeling, and safety and efficacy in the context of reduced NME exposure. The in vitro information seems to be widely used for prioritizing clinical DDI studies in the development programs, providing guidance for clinical study designs, and for recommending the inclusion/exclusion criteria and contraindicated medications.

A decision tree approach based on the available in vitro induction data and the therapeutic concentrations of NME is recommended for deciding whether a clinical DDI study is needed (Fig. 2). In many cases, negative findings from early in vitro studies can eliminate the need for clinical DDI investigations. As discussed elsewhere in this article, this decision is not straightforward for isoforms other than CYP3A4. It is recommended that a case-by-case analysis be made for other isoforms, keeping in view the magnitude of predicted interaction, the therapeutic indices of potentially coadministered drugs that are metabolized by the P450 isoform of interest, and other factors that may affect safety and tolerability during coadministration.

In studying an investigational drug as an inducer, the choice of substrates for initial in vivo studies depends on the P450 enzymes affecting the interacting drug (Table 2). If the initial study shows that the NME induces metabolism of a sensitive substrate, further studies using less sensitive substrates, based on the likelihood of coadministration, may be useful. If the initial study is negative with the most sensitive substrate, then interaction with less sensitive substrates can be assumed to be negative. Furthermore, if the NME is not found to be an inducer of CYP3A4 in vitro or in a clinical DDI study, it can be assumed that it will not affect other enzymes that are inducible via the PXR-mediated pathway (e.g., CYP2C8, CYP2C9, and CYP2C19).

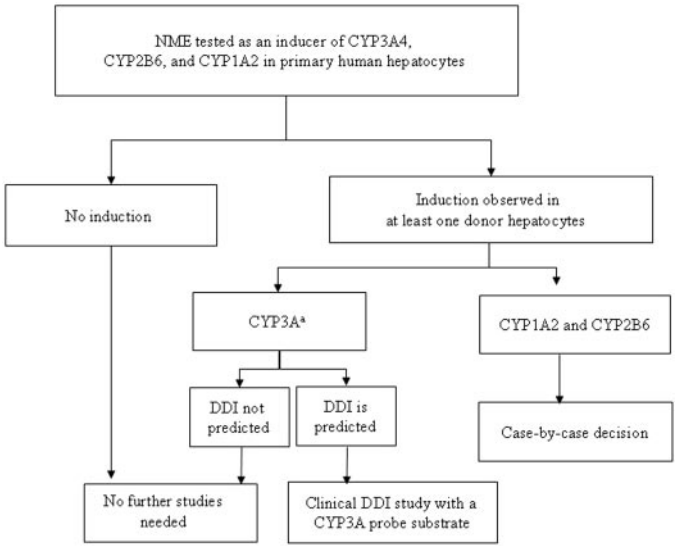

FIG. 2. Decision tree for clinical induction studies with a new molecular entity. [a], An empirical assessment or prediction based on mathematical or correlation-based approaches described in the IVIVC section.

Clinical Study Design Considerations. The HIV protease inhibitor ritonavir offers a good example of the need for a well thought out clinical study design to evaluate the inductive responses of an NME. Ritonavir is not only a potent inhibitor but also an inducer of CYP3A (Hsu et al., 1998). The dual inhibition and induction of CYP3A may produce complex and time-dependent pharmacokinetic interactions with other drugs. The net effect of ritonavir on CYP3A-mediated metabolism in vivo represents a balance of inhibition and induction, which cannot be easily predicted (Greenblatt et al., 1999). For such compounds, inhibition will probably dominate initially, whereas on more extended exposure, induction may partially or completely offset inhibition.

The clinical DDI study should be designed with the aim of providing specific recommendations regarding the clinical significance of the interaction based on what is known about the dose response and/or the pharmacokinetic/pharmacodynamic relationship for either the NME or approved drugs used in the study. The selection of a particular study design depends on a number of factors for both the substrate and interacting drug such as 1) the use of the substrate and/or NME in an acute or chronic condition, 2) the therapeutic window of substrate and NME, and 3) the pharmacokinetic-pharmacodynamic characteristics of the substrate and NME.

Study population. The choice of the population in the clinical DDI study will largely be driven by the indication and nature of the likely induction. The ideal population to conduct such studies is the healthy volunteers, because they offer the greatest flexibility for assessments and monitoring. However, in certain indications such as oncology and/or when an NME cannot be administered to healthy volunteers, a typical patient population may be needed. The survey responses are in agreement with this practice (77% using healthy volunteers), except for oncologic and cytotoxic NMEs. Sometimes it is possible to assess the clinical consequences of induction in the context of a clinical study (Fine et al., 2004).

Dose selection. For both the substrate and NME, testing should maximize the possibility of finding an interaction. For this reason, the maximum planned or approved dose and shortest dosing interval of the NME should be used, and the duration of the treatment should be sufficient to reach the steady state or a clinically relevant regimen. This survey indicates that almost all the responding companies (93%) dose NME for at least five days or to steady state when conducting a

TABLE 2

Recommended in vivo substrates for P450 induction studies

Probe Substrate to Evaluate NME As an Inducer		
P450 Isoform	Substrate[a]	Recommended Dose and Route
CYP1A2	**Theophylline**, caffeine	200 mg oral
CYP2B6	**Bupropion**,[b] efavirenz	150 mg oral
CYP2C8	**Rosiglitazone**, repaglinide	8 mg oral
CYP2C9	**Warfarin**, tolbutamide	2 to 5 mg oral
CYP2C19	**Omeprazole**, esomeprazole, lansoprazole, pantoprazole	20 mg oral
CYP2E1[b]	**Chlorzoxazone**	500 mg oral
CYP3A4/3A5	**Midazolam,** buspirone, felodipine, lovastatin, eletriptan, sildenafil, simvastatin, triazolam	10 mg oral

[a] Substrates in bold are preferred substrates.
[b] Assessment of hydroxybupropion exposure.

clinical DDI study. Additional factors that may be taken into consideration include the existence of major and/or pharmacologically active metabolites of the NME, dose- and time-dependence of NME pharmacokinetics, and any safety issues that may ensue when NME is dosed in combination with a probe substrate.

Assessments. Changes in pharmacokinetic parameters together with a good understanding of dose/concentration and concentration/response relationships for both desirable and undesirable drug effects in the general population or in specific populations can be used to assess the clinical relevance of the drug-drug interaction. Pharmacodynamic endpoints, if available, may be useful complements to pharmacokinetic data in understanding the clinical significance of interactions (e.g., biomarkers, prothrombin time for warfarin, and blood glucose assessments with antidiabetic drugs).

Data analysis and reporting. Results of drug-drug interaction studies should be reported as 90% confidence intervals about the geometric mean ratio of the observed pharmacokinetic measures with and without the interacting drug. The number of subjects required for a given drug-drug interaction study will depend on how small an effect is clinically important to detect or rule out, the inter- and intrasubject variability in pharmacokinetic measurements, and possibly other factors or sources of variability that are not well recognized.

Recommendations for Clinical Drug-Drug Interaction Studies. *NME as an inducer.*

- Based on in vitro induction study data and the likely therapeutic concentration, follow the decision tree approach to assess whether a clinical DDI is needed.
- Choose the most sensitive probe substrate for clinical DDI study (Table 2).
- NME dosed to steady state or a clinically relevant regiment at the highest dose and shortest dosing interval intended for the marketing approval.
- Assess pharmacokinetics of probe before and after NME treatment, and document NME exposure.
- Data analysis and interpretation should include clinical consequences of induction relative to the therapeutic index of the substrates of the affected enzyme.

Alternate assessments. The measurement of urinary 6β-hydroxycortisol/cortisol urinary ratio provides a simple noninvasive method to monitor CYP3A4 induction, as demonstrated with many drugs including carbamazepine, a known inducer of CYP3A4 (El Desoky et al., 2005). However, 6β-hydroxycortisol/cortisol urinary ratio and midazolam clearance were reported to be poorly correlated, probably due to high intraindividual variability of the 6β-hydroxycortisol/cortisol urinary ratio compared with midazolam clearance (Chen et al., 2006). Based on the observation that CYP3A4 inducer carbamazepine increases plasma 4β-hydroxycholesterol levels, it has been suggested that this could be a biomarker for CYP3A4 induction in humans (Diczfalusy et al., 2008; Wide et al., 2008). Survey responses indicate limited use of these techniques.

The Erythromycin Breath Test (Watkins, 1996) measures liver CYP3A4 catalytic activity, and because CYP3A4 represents the major metabolic pathway for many therapeutics, this test provides a phenotypic measurement of enzymatic activity and the effect of putative inducers. A recent clinical study suggests that the role of uptake and efflux transporters in the disposition of erythromycin could complicate the interpretation of the results (Frassetto et al., 2007). A similar methodology has been tested successfully for CYP1A2-mediated caffeine *N*-demethylation (Rost et al., 1992). Survey responses indicate occasional use of these techniques.

A novel approach to assess in vivo measurement of P450 mRNA expression in peripheral blood lymphocytes as a predictor of enzyme induction has not been successful (Haas et al., 2005). If successfully developed in future, an approach similar to this effort would be of enormous value in identifying the potential for induction-based DDI and dose adjustments based on simple diagnostic methods.

Some groups have recommended a cocktail of several isoform-specific P450 substrates as an alternate method of conducting clinical DDI studies (Tomalik-Scharte et al., 2005; Yeh et al., 2006). Although this approach may be a good investigational tool, it may not be an appropriate approach for labeling purposes when an interaction does occur.

Alternate Models and New Technologies. Although cultured human hepatocytes have been accepted as the "gold standard" for nonclinical drug interactions studies, various attempts are being made with alternate methods for assessing P450 induction. The present survey included questions about the appropriateness and adaptation of several new technologies.

Human liver slices. Precision-cut liver slices have been proposed as an alternate model to hepatocytes for assessing metabolism of NMEs in vitro. Because cellular architecture is intact in liver slices, it is thought that this may be a better representation of the in vivo situation. This model has not been widely used for enzyme induction studies due to various shortcomings related to fresh liver availability and difficulties in maintaining enzymatic activities for prolonged periods, although progress has been reported (Lupp et al., 2002; Persson et al., 2006). In a study conducted by Olinga et al. (2008), it was demonstrated that AhR, CAR, and PXR-mediated induction of drug-metabolizing enzymes (both Phase I and II) as well as drug transporters were inducible by prototypical inducers of these pathways in human liver slices.

Animal models. The interspecies differences in homology, expression, regulation, and substrate specificity of P450s is well known

(Martignoni et al., 2006). The time-dependent exposure decreases of an NME in nonclinical multiple dose toxicology studies are generally due to autoinduction of NME metabolism. However, due to interspecies differences, this observation may not translate to humans. Based on enzyme homology, overlapping substrate specificities, and in vivo observations, rhesus monkey has been proposed as an acceptable model for CYP3A-mediated drug interactions (Prueksaritanont et al., 2006). However, the survey responses indicate that only one company uses monkey as a model to predict human P450 induction.

Chimeric animal models. Transplantation of human hepatocytes into mouse liver to generate chimeric mice with humanized liver has been successful (Tateno et al., 2004). In such chimeric mice, the expression of the major P450 isoforms and their catalytic activities have been found to be similar to those of the donor hepatocytes. In studies conducted by Katoh et al. (2005, 2008), rifampicin increased CYP3A4 mRNA, protein, and catalytic activity (dexamethasone 6-hydroxylation) by 8- to 22-, 3- to 10-, and 5- to 12-fold, respectively. In the same study, 3-MC increased CYP1A2 mRNA and protein expression by 2- to 9-fold and 5-fold, respectively. The authors concluded that the chimeric mouse may be a useful model to estimate and predict the in vivo induction of P450s in humans. The authors also point out that chimeric mice could be used to proliferate human hepatocytes at a low cost, which would be an advantage in countries where the availability of human hepatocytes is scarce.

Humanized animal models. In the last few years, humanized mouse models expressing human genes of P450 isoforms and nuclear receptors, or a combination of both, have been developed and evaluated (Xie et al., 2000; Xie and Evans, 2002; Gonzalez and Yu, 2006; Felmlee et al., 2008). Although the results are promising, the general feeling of the survey respondents is that these models "need more work" for routine use, although they may be helpful for mechanistic studies in vivo.

Concluding Remarks. This survey of PhRMA member companies clearly demonstrated that P450 induction has gained wide acceptance as an important factor for consideration during drug discovery and development. Many companies have implemented assays for nuclear receptors, most notably PXR, for early detection of the potential for induction. The greater availability of fresh and cryopreserved hepatocytes in the United States has facilitated the emergence of this in vitro model as the gold standard for evaluating the potential for P450 induction. The accumulation of considerable data from in vitro hepatocyte induction studies, and the in vitro and in vivo correlations, albeit semiquantitative at this time, has eliminated the need for unnecessary clinical drug-drug interaction studies for compounds that are not inducers in vitro. The acceptance of the in vitro data by clinical groups in pharmaceutical companies as well as regulatory agencies as a reliable predictor of clinical drug-drug interactions due to P450 induction has been a notable development.

in experiments, emergence of highly sensitive and rapidly deployable quantitative assays for measurement of mRNA (e.g., TaqMan) and isoform-specific probe catalytic activities (e.g., liquid chromatography/tandem mass spectrometry) has made a tremendous impact on the conduct of these assays, which have previously been time-consuming and cumbersome. In addition, cryopreservation and immortalization of hepatocytes has enhanced the flexibility of conducting these assays. The burden has now shifted from the experimental portion to data interpretation and extrapolation to the in vivo situation. As described in the IVIVC section, notable progress has been made in this area recently. It should be clearly stated that a simple experimental approach, for example NME tested at concentrations spanning clinical concentrations, with comparison to a positive control at a single concentration, would be sufficient for decision making purposes, and such data will provide enough information to help in the design of a clinical program. If a company feels that a more quantitative prediction is necessary, a more elaborate set of in vitro studies may be performed and an in vitro-in vivo correlation may be attempted, but such strategies are not needed as a routine practice.

One of the important considerations in interpreting in vitro data is to take into account the context of the clinical situation. As discussed in the IVIVC section, there is generally an acceptable correlation between in vitro and in vivo observations for CYP3A4 induction. This result is to some extent due to the wealth of information available for this enzyme. However, for CYP1A2 and CYP2B6, that is not the case. As noted elsewhere in this document, the example of omeprazole demonstrates the importance of understanding the relationship between in vitro-derived induction information in the context of systemic exposure of the NME.

This article does not address induction of minor P450 isozymes such as CYP2E1, Phase II enzymes such as uridine glucuronosyltransferases, and influx/efflux transporters. On a case-by-case basis, it might be prudent to assess the inducibility of these enzymes and/or transporters if it is suspected that a clinically relevant DDI will occur between the NME and a coadministered medication that is primarily cleared via these pathways.

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